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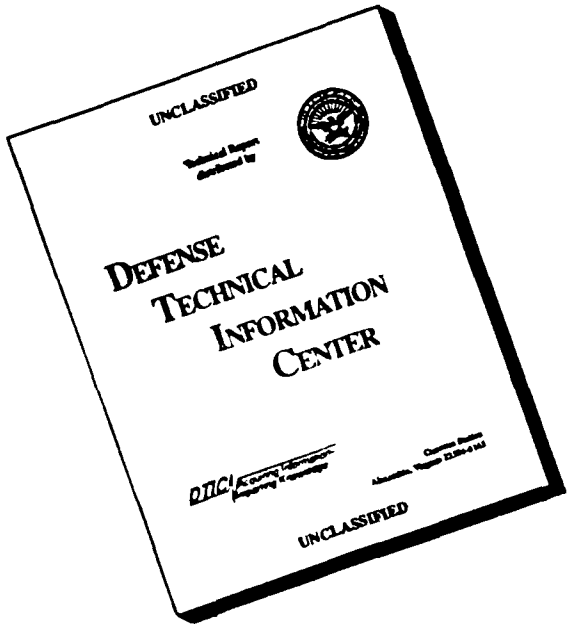

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Introduction to Functional Cell Assays

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The flow cytometer can be used to measure a variety of functional parameters that are of increasing interest to cell biologists. Some of these parameters are already of interest in the clinical laboratory and some will become clinically relevant in the near future. The recent development of a number of new fluorescent probes now permits the measurement of various intracellular free ion concentrations, membrane characteristics, indicators of cellular metabolism, and intracellular glutathione. Most previously available techniques to measure these cellular parameters determined the mean value for a mixed population of cells. The flow cytometer has the unique capacity to permit the measurement of physiologic parameters in large numbers of living single cells; it allows correlation with other parameters such as immunophenotype and cell cycle; and, finally, it reveals heterogeneity within the cell population.

As an introduction to several more detailed presentations elsewhere in this volume that describe the clinical application of probes of cellular physiology, this discussion will introduce several measurements of the greatest current interest.

INTRACELLULAR IONIZED CALCIUM

Ionized calcium has an important role as a mediator of transmembrane signal transduction, and elevations in intracellular ionized calcium concentration ($[Ca^{2+}]_i$) regulate diverse cellular processes. Measurement of $[Ca^{2+}]_i$ in living cells is thus of considerable interest to a broad range of investigators.

Calcium influx is thought to be initiated either by membrane depolarization that opens voltage-gated channels or by the binding of ligands to receptor-operated channels. The binding of agonist to its specific membrane receptor activates enzymatic processes that result in the activation of phospholipase C. Phospholipase C causes the hydrolysis of a membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2), which yields a water-soluble product, inositol 1,4,5-trisphosphate (IP3), and a lipid, 1,2-diacylglycerol (DAG). IP3 then causes the release of calcium from intracellular stores, whereas DAG in conjunction with calcium ions

ctivates protein kinase C. Thus, a single agonist can result in the production of at last two "second messengers", making this pathway a unique, bifurcating system. Calcium is therefore a "third messenger" that controls numerous cellular processes, ctivating a broad variety of enzyme systems, both as a cofactor and in conjunction with the calcium-binding protein, calmodulin.

Indicators of Intracellular Ionized Calcium Concentration

Until 1982, it was not possible to measure $[Ca^{2+}]_i$ in small intact cells; measurements were restricted mostly to large invertebrate cells where the use of microelectrodes was possible. $[Ca^{2+}]_i$ was first measured in diverse populations of cells with the development of quin2.[1] The indicator was easily loaded into small intact cells using the acetoxymethyl ester of the dye.[2] This uncharged form diffuses freely into the cytoplasm where it is hydrolyzed by esterases to yield the tetraanionic form of the dye, which is trapped inside the cell. Unfortunately, quin2 has several disadvantages that limit its application to flow cytometry.[3] A relatively low extinction coefficient and quantum yield have made detection of the dye at low concentrations difficult; at higher concentrations, quin2 itself buffers the $[Ca^{2+}]_i$. Subsequently, Grynkiewicz *et al.*[4] described a new family of highly fluorescent calcium chelators that overcome most of the aforementioned limitations. One of these dyes, indo-1 (1-[2-amino-5-(6-carboxylindol-2-yl)-phenoxy]-2-[2'-amino-5'-methylphenoxy]ethane *N,N,N'N'*-tetraacetic acid), has spectral properties that make it especially useful for analysis with flow cytometry. In particular, indo-1 exhibits large changes in the fluorescent emission wavelength upon calcium binding. Use of the ratio of the intensities of fluorescence at two wavelengths (approximately 400 nm and 500 nm) allows calculation of $[Ca^{2+}]_i$ independent of variability in cellular size or intracellular dye concentration. The ratiometric approach thus gives considerable accuracy to this analysis, allowing, for example, the basal $[Ca^{2+}]_i$ to be seen to be constrained within narrow limits (FIGURE 1). The only significant drawback to the use of indo-1 is the requirement for ultraviolet (UV) excitation.

A practical alternative to indo-1 became available upon the description of a fluorescein-based, calcium-sensitive probe, fluo-3.[5] This dye exhibits an increase in fluorescence intensity with increasing $[Ca^{2+}]_i$. The use of fluo-3 permits, for the first time, the simultaneous use of other UV-excitable probes for flow cytometry, such as those used for cell cycle analysis or measurement of intracellular glutathione, allowing correlation of these parameters with calcium responses. The primary disadvantage of fluo-3 is that it does not have fluorescence properties that allow ratiometric determinations. Therefore, calibration on a flow cytometer is more complicated because the signal is proportional to cell size and dye concentration as well as to $[Ca^{2+}]_i$. In addition, the ability to measure responses in subsets of cells is more limited because the broad distribution of fluorescence intensities of unstimulated cells often results in an overlapping distribution of the values from stimulated and unstimulated cells (FIGURE 1). This problem can be minimized by the simultaneous use of a second dye that serves as an indicator of the magnitude of dye loading in an individual cell.[6]

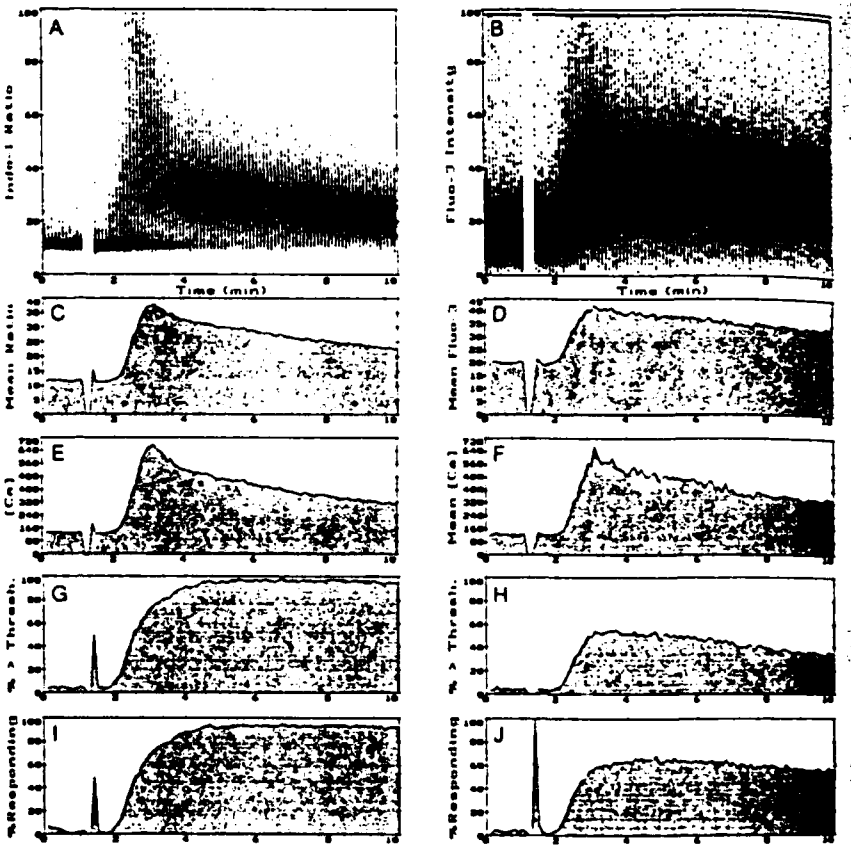

FIGURE 1. Comparison of indo-1 and fluo-3 measurement of $[Ca^{2+}]_i$ and methods of display of kinetic data. Human peripheral blood lymphocytes (PBL) were loaded simultaneously with 3 μg/mL indo-1 and 3 μg/mL fluo-3, stained with PE-CD4 mAb, and then stimulated with anti-CD3 antibody (10 μg/mL G19-4, Jeff Ledbetter, Oncogen, Seattle, Washington) at approximately 1 minute after the start of analysis (note the gap in data acquisition). Violet and green indo-1 emission (UV excited) and green fluo-3 and orange PE emission (488-nm excited) were collected simultaneously for each cell. Results were gated from PE-$CD4^+$ cells. In panels A and B, the results are displayed as a "dot plot" where the x-axis is time and the y-axis is the indo-1 ratio or the fluo-3 fluorescence intensity. The mean indo-1 ratio versus time is shown in panel C and the fluo-3 intensity versus time is shown in panel D. The data were converted to calcium concentration versus time by calibration using measured constants for indo-1 (panel E) and by calibration with buffer solutions for fluo-3 (panel F). Whereas the shapes of the curves for the mean indo-1 ratio (C) and the fluo-3 intensity (D) are different (due to the different values of K_m for indo-1 and fluo-3), the two measurements are essentially identical once converted to $[Ca^{2+}]_i$. The percentages of cells responding with $[Ca^{2+}]_i$ elevated beyond two standard deviations above the mean of the cells before antibody stimulation ("percent cells above threshold") are plotted for indo-1 (panel G) and fluo-3 (panel H). The percentages of responding cells calculated by cumulative curve subtraction are plotted in panels I and J for indo-1 and fluo-3, respectively. Data analysis was performed with software written by one of the authors (P. S. Rabinovitch) ("MultiTime", Phoenix Flow Systems, San Diego, California).

Applications of the Flow Cytometric Analysis of $[Ca^{2+}]_i$

A common extension of analysis of $[Ca^{2+}]_i$ is the simultaneous use of additional fluorochromes for the determination of cellular immunophenotype. This allows alterations in $[Ca^{2+}]_i$ to be examined in specific immunophenotypic subsets (FIGURE 2). Fluorescein isocyanate (FITC)– and phycoerythrin (PE)–conjugated antibodies can be used with indo-1, and PE and allophycocyanin (APC) (or other red-excited dyes) can be used with fluo-3. Numerous examples of the analysis of $[Ca^{2+}]_i$ in immunophenotypically defined subsets have been described (reviewed in reference 7).

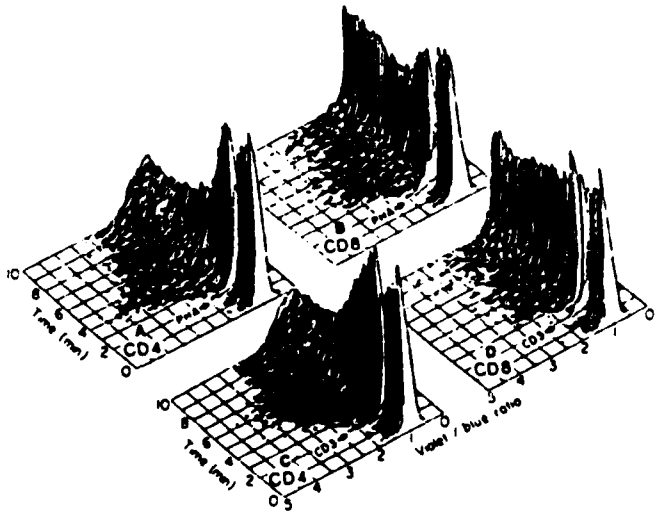

FIGURE 2. Analysis of $[Ca^{2+}]_i$ in human PBL stained with indo-1 and a cocktail of PE-labeled antibodies that stain all cells but $CD4^+$ (A,C) or $CD8^+$ (B,D). Analyses were gated to exclude all PE-labeled cells, a reciprocal staining strategy that avoids antibody binding to CD4 and CD8 receptors.[9] Cells were stimulated with 30 μg/mL PHA (A,B) or 10 μg/mL CD3 (G19-4) (C,D). The indo-1 ratio is displayed versus time as an isometric plot where the z-axis is the cell number. Note that $[Ca^{2+}]_i$ responses in $CD4^+$ cells are of higher magnitude than those of $CD8^+$ cells.

The flow cytometric assay of cellular calcium concentration has already been applied to a wide variety of cells, providing interesting and sometimes unexpected results. Examples of the initial applications of the technique are presented in recent reviews.[7,8] One of the first observations that was made readily quantifiable by the flow cytometric analysis was that there is great heterogeneity in the response of lymphocytes to mitogenic agonists. Using simultaneous immunofluorescence, some of this heterogeneity can be shown to be related to immunophenotypic subsets; for instance, $CD4^+$ cells show more vigorous responses to lectins and CD3 than do $CD8^+$ cells (FIGURE 2).[9,10] Considerable use of this approach has been made in the demonstration of differences between $[Ca^{2+}]_i$ activation requirements of different cell subsets

and subset specificities of activation pathways. The effects of antibody binding to cell surface molecules, sometimes a complication in labeling experiments (see legend to FIGURE 2), have been extensively employed to analyze signaling mechanisms, and augmented or even new relationships have been probed by cross-linking antibodies on the cell surface.[11,12] Flow cytometric measurements with indo-1 have been performed to date with all nucleated blood cell types. Applications of fluo-3 have been reported with most types, with reports appearing at a rapidly increasing pace.

The combination of sensitivity, reliability, and ability to analyze large numbers of cells within cell subsets has made the flow cytometric assay of $[Ca^{2+}]_i$ the preferred technique for a broad spectrum of research applications. As a consequence of the success of this research, there are now many exciting potential clinical applications of the flow cytometric assay of cellular calcium concentration. The contribution by June *et al.* in this volume describes some of these developing clinical applications.

INTRACELLULAR pH

The pH_i of mammalian cells is ~7.2 and it appears to be closely regulated. In mammalian cells, pH_i is controlled by at least three mechanisms, including Na^+_o/H^+_i exchange, sodium-dependent $Cl^-_o/HCO^-_{(3)i}$, and $HCO^-_{(3)o}/Cl^-_i$ exchange. Acid extrusion is primarily accomplished by the Na^+/H^+ antiport and by sodium-dependent $Cl^-_o/HCO^-_{(3)i}$, whereas $HCO^-_{(3)o}/Cl^-_i$ exchange has the major role for base extrusion. All of these mechanisms appear to be stimulated by a variety of growth factors and by phorbol esters, presumably through the activation of protein kinase C.

Indicators of Intracellular pH

Until recently, the most commonly used probes were modifications of fluorescein, with fluorescein diacetate being the first-generation pH probe, followed by carboxyfluorescein diacetate (COFDA). Both of these dyes are limited by relatively poor retention inside loaded cells. An improved fluorescein probe is 2',7'-bis-carboxyethyl-5(6)-carboxyfluorescein (BCECF). As with indo-1 and fluo-3, BCECF is loaded into cells using the acetoxymethyl ester; after hydrolysis, it has a negative charge of −4 or −5 and therefore leaks more slowly than COFDA. The pK_a of BCECF, 6.98, is near the pH_i of resting cells and there is a pH-dependent shift in the excitation wavelength, making it possible to use the ratio of fluorescence signals to correct for differences in loading and cell size. In addition, BCECF fluorescence excited at 450 nm is pH-independent, whereas fluorescence at 500 nm is pH-dependent, allowing ratiometric fluorescence emission analysis (TABLE 1). In both cases, the magnitude of pH-dependent ratio shifts is relatively modest[13] and these dyes are largely supplanted by newer probes. The most useful of several UV-excited pH probes that have been developed is 1,4-diacetoxy-2,3-dicyanobenzene (ADB). The cell-permeant ADB is hydrolyzed and trapped intracellularly to yield 2,3-dicyanohydroquinone (DCH).[14] Ratiometric determinations of pH are possible using a single excitation source by measuring the fluorescence emission at 429 nm and 477 nm (TABLE 1). As with the measurement of $[Ca^{2+}]_i$, many laboratories will find the requirement for UV excitation to be the primary limitation for the use of this probe.

Recently, a useful probe for pH_i measurement named SNARF-1 (SemiNaphtho-RhodaFluor) has been introduced.[15] SNARF-1 has convenient excitation spectra (488 or 514 nm) and exhibits large changes in pH-dependent fluorescence. The emission of SNARF-1 in acid is maximal at 587 nm and the basic form emits maximally at 636 nm; there is an isosbestic point at 610 nm. The ratio of orange and red emission is used as the pH_i indicator. This provides the advantages of the ratiometric determination, as described previously.

Applications of Analysis of pH

As previously mentioned, the fluorescence emission properties of SNARF-1 allow simultaneous excitation of FITC probes using the same 488-nm laser. Analysis of pH_i in immunophenotypically defined cell subsets using FITC-conjugated mAb is thus very straightforward. Similarly, single-laser $[Ca^{2+}]_i$ measurements with fluo-3 are easily performed simultaneously with measurements of pH_i. When a second UV laser is available, the $[Ca^{2+}]_i$ measurement with indo-1 is optimal.[16]

TABLE 1. Fluorochromes for Ratiometric Determination of pH Using Flow Cytometry

Probe	pK'_a	Fluorescence (nm)	
		Excitation	Emission
bis-carboxyethyl-carboxyfluorescein acetoxymethyl ester (BCECF AM)	6.98	ratio 439/490 488	535 ratio 520/620
diacetoxy-dicyanobenzene (ADB); yields dicyanohydroquinone (DCH) after deesterification	8.0	~350	ratio 425/540
carboxy-SNARF-1-acetoxymethyl acetate	7.50	488, 514	ratio 575/670

Because of the large shifts in pH_i that take place upon activation of granulocytes, studies of intracellular pH in the analysis of granulocyte function are probably the most interesting in the clinical laboratory today. These are discussed by G. Valet in this volume.

INTRACELLULAR GLUTATHIONE

Glutathione (glutamylcysteinylglycine, GSH) is an important antioxidant tripeptide thiol that is involved in the scavenging of toxic oxygen products.[17,18] In addition, GSH is involved in a number of other important reactions in the cell, including conjugation of xenobiotics, amino acid transport, and deoxyribonucleotide synthesis.[17] GSH is also important for the maintenance of cellular thiol redox status, and its redox state has been proposed to be a major determinant of the functioning of a number of enzymes and in the integrity of cytoskeletal proteins. These roles for GSH

have important consequences upon cell physiology and mitogenic activation. In general, augmenting cellular GSH with cysteine delivery agents, such as *N*-acetylcysteine or oxathiazolidine carboxylic acid, or with glutathione esters has a positive effect on cell growth, whereas depletion of GSH inhibits many cellular functions.[17] GSH is known to influence cell growth and replication at various levels, including G_1/S-phase transition[19] and very early events in mitogen-induced cell activation.[20] GSH has also been shown to affect the responsiveness of cells to various cytokines, for example, the action of IL-2 in lymphocytes.[21,22]

The activity of redox-responsive oncogene products and DNA-binding factors responsible for gene regulation may also be affected by the GSH status of the cell. For instance, the interactions of c-*fos* and c-*jun* products are known to be redox-sensitive.[23] Another example is that of NFkB. Roederer and colleagues[24] have shown that this nuclear binding factor can be inhibited from binding to regulatory DNA-binding domains by *N*-acetylcysteine, a drug that increases the GSH level of cells.

Indicators of Intracellular GSH

Several years ago, Durand and Olive[25] published a review of fluorescent indicators for thiols, including GSH, that might prove useful for flow cytometric purposes. The problem with all of the dyes reviewed was one of specificity for GSH. A major advance in the measurement of GSH by flow cytometry was made by Rice *et al.*,[26] who used monochlorobimane (MCB) to detect changes in the GSH status of individual normal and tumor cells. This dye owes its specificity for GSH (see FIGURE 3) to the fact that it is conjugated to GSH by glutathione-*S*-transferases and has

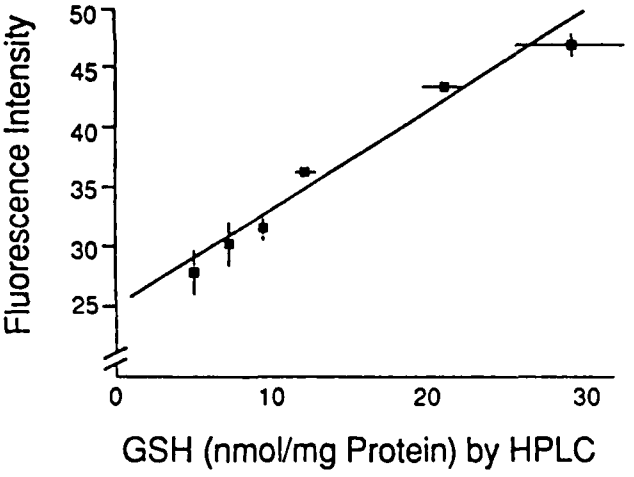

FIGURE 3. Correlation between GSH content measured by HPLC and MCB flow cytometry. Chinese hamster V79 cells were treated with 0, 1, 3, 6, 9, or 12 mM BSO for 12 hours. Cells were then processed for flow cytometry (45 μM MCB for 10 minutes at 37°C) or for HPLC determination of the total reduced GSH content (from reference 44, with permission from the publisher).

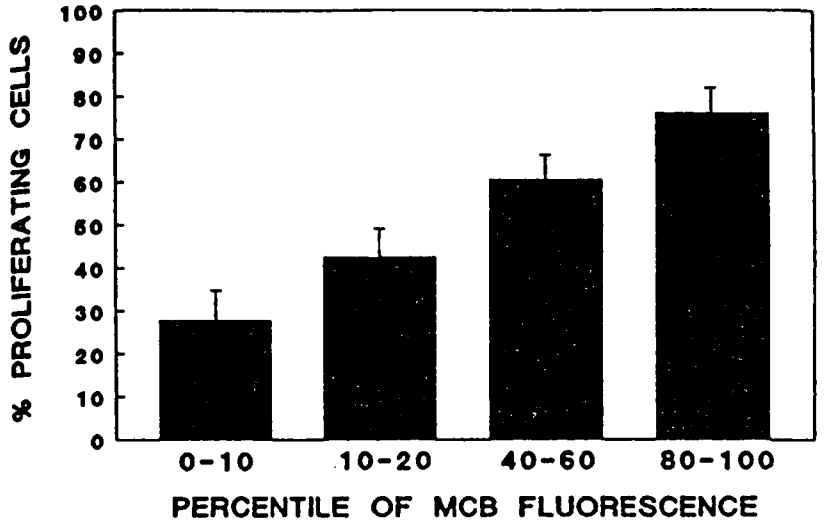

FIGURE 4. Proliferation of human $CD4^+$ lymphocytes sorted on MCB fluorescence intensity. Human lymphocytes isolated from peripheral blood by density gradient centrifugation were stained with MCB (60 μM for 15 minutes at 37 °C) and PE anti-CD4 mAb. $CD4^+$ cells were then sorted according to their MCB fluorescence for the lowest 10%, the 10th to 20th percentile, the 40th to 60th percentile, and the highest 20% of the fluorescence histogram. Cells were then plated into 96-well plates precoated with anti-CD3 mAb in BrdU-containing medium. After three days, cells were harvested and assessed for proliferation by the BrdU, Hoechst method.[45] There is a direct correlation between the MCB fluorescence intensity of the unstimulated cells and their subsequent ability to proliferate.

relatively low nonenzymatic reactivity towards GSH and other thiols.[27] As examples of the application of the measurement of GSH in viable cells using MCB, FIGURES 4 and 5 show that the proliferation and $[Ca^{2+}]_i$ responses of sorted $CD4^+$ T cells are proportional to their basal GSH content.

Another reagent, chloromethylfluorescein diacetate (CMFDA), has recently been described for measuring intracellular GSH.[28] Intracellular CMF is presumably metabolized in a manner similar to MCB, yielding a GSH-MF fluorescent conjugate. The advantage provided by this dye is that the excitation and emission characteristics are similar to fluorescein once conjugated to GSH, allowing the use of 488-nm excitation. The principal disadvantage with this dye is that it is apparently less specific for GSH than MCB.[28]

Applications of Analysis of GSH

In the clinical laboratory, GSH has recently received attention because of its role in a number of pathological disease states including tumor cell resistance to chemotherapeutic agents (see the contribution by D. W. Hedley in this volume) and idiopathic pulmonary fibrosis.[29] In addition, considerable recent attention has been

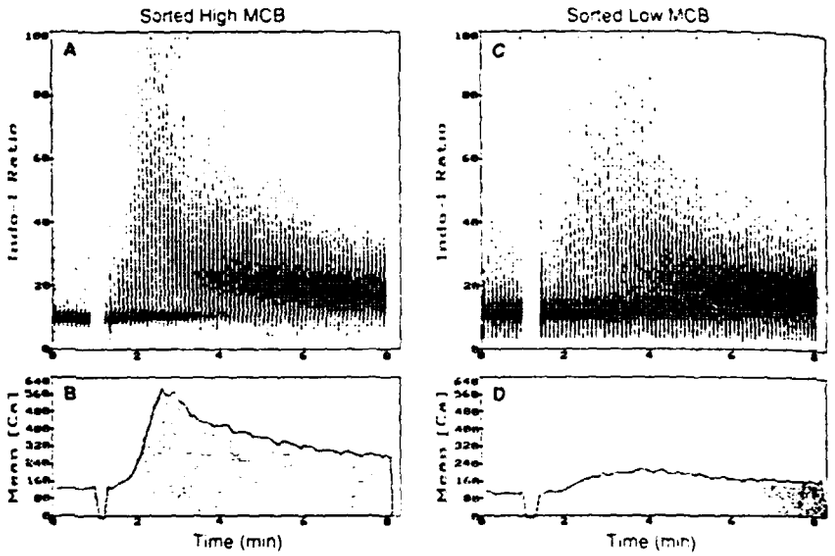

FIGURE 5. Effect of intracellular GSH content on anti-CD3 mAb-stimulated transmembrane signal transduction in $CD4^+$ human T lymphocytes. Human PBL were stained with 60 μM MCB and PE-anti-CD4 mAb. $CD4^+$ cells were sorted as the upper and lower 30% of the MCB fluorescence distribution, allowed to recover for 24 hours, and then either loaded with indo-1 AM or restained with MCB. Cells restained with MCB retained their originally sorted MCB fluorescence differences (not shown). Cells loaded with indo-1 were analyzed for $[Ca^{2+}]_i$ content following stimulation with anti-CD3 mAb (as described in FIGURE 1). Panels A and C show the kinetics of $[Ca^{2+}]_i$ transients in cells sorted on high and low GSH content, respectively. There is a diminished response in cells with low GSH content. Panels B and D show the mean $[Ca^{2+}]_i$ calculated from the data presented in panels A and C, respectively. There is a higher mean and peak calcium response in the high GSH-sorted cells than in the low GSH-sorted cells.

directed towards the analysis of GSH in acquired immune deficiency syndrome.[24,30-33] This subject is addressed in detail by Roederer *et al.* in this volume.

ANALYSIS OF MEMBRANE POTENTIAL

Resting cells maintain large gradients between the intracellular and extracellular concentrations of a variety of ions, including Ca^{2+}, K^+, Na^+, and Cl^-. Potassium ions, for example, are concentrated within cells by the action of the Na-K-ATPase. The relative permeability of the membrane to K^+ ions is greater than that of other ions; the leakage of K^+ ions establishes an electron countergradient and the cytoplasm becomes electronegative with respect to the external medium. This K^+ electrochemical gradient is the most significant contribution to the negative membrane potential of most mammalian cells. Maintenance of a large negative transmembrane potential has been postulated to be a control mechanism to arrest cells in an inactive stage and changes in cell membrane potential that occur in various cell types rapidly after

binding of ligands to transmembrane receptors have been suggested to be mediators of subsequent physiologic cellular responses. Detailed investigation of the membrane potential in small cells has been made feasible by the development of potential sensitive indicator probes. These probes are charged lipophilic molecules; they partition between the cell and the surrounding medium according to the Nernst equation: $C_c/C_o = e^{-nF\epsilon/RT}$, where C_c and C_o are the cytosolic and extracellular indicator concentrations; n is the charge of the indicator; ϵ is the membrane potential; and F, R, and T are the Faraday and gas constants and temperature. For a cationic indicator, the cellular concentration falls as the membrane potential declines towards zero and it rises if the cell hyperpolarizes (i.e., the cytosol becomes more electronegative with respect to the medium).

Cyanine Dyes

A family of cationic cyanine dyes were described by Hoffmann and Laris[34] to be useful indicators for cells in solution, in the manner described earlier. These fluorescent dyes have a single negative charge delocalized over an extensive pi-electron system in a highly symmetric molecule. Sims *et al.*[35] introduced the shorthand nomenclature $DiYC_n(2m+1)$ for these dyes, where the "Y" member of the ring structure may be oxygen (O), sulfur (S), or isopropyl (I). The length of the alkyl side chains, "n", affects the lipid solubility and "m", the number of methene groups, affects the fluorescence spectral characteristics. The use of the highly fluorescent dyes shown in TABLE 2 with flow cytometry allows adequate fluorescence signal detection of single cells with dye concentrations below 10^{-7} M. Under these conditions, hyperpolarization is accompanied by increased cellular fluorescence and depolarization is accompanied by decreased fluorescence. These low dye concentrations also help to minimize the toxicity of the cyanine dyes.

Oxonol Dyes

The oxonol dyes are chemically unrelated to the cyanine dyes, but are similarly symmetric, membrane-permeant molecules with a highly delocalized charge. This charge is negative, in contrast to the cyanine dyes, so that the changes in partitioning

TABLE 2. Dyes for Flow Cytometric Analysis of Membrane Potential

	Excitation (nm)	Emission (nm)
Cyanine Dyes		
$DiOC_5(3)$	488	510–540
$DiOC_6(3)$	488	520–540
$DiSC_3(5)$	568, 633	>590, >680
$DiIC_4(3)$	488, 514	540–580
Oxonol Dyes		
$DiBaC_4(3)$	488	510–540
$DiBaC_4(5)$	568–595	610–640
$DiSBaC_2(3)$	568	590–630

in response to altered membrane potential are in the opposite direction to cyanine dyes: depolarization of the membrane transfers the anion from the external medium onto binding sites within the cell and hyperpolarization results in dye exclusion and decreased cellular fluorescence. For flow cytometry, the properties of the oxonol dyes are especially attractive: analysis of cells takes place almost completely apart from the dye in the external medium (increasing the signal-to-noise ratio). In addition, the lower proportion of bound dye increases the buffering of the external dye concentration and the negative charge of the dye forces exclusion from the highly negatively charged mitochondria, minimizing a complication encountered with cyanine dyes.

Application of the Study of Membrane Potential by Flow Cytometry

An increasing fraction of studies of membrane potential are being performed by flow cytometry, taking advantage of the sensitivity of this methodology, the ability to recognize heterogeneity in cellular responses, and the opportunities for multiparameter analysis. These applications have been recently reviewed.[7,36]

Flow cytometric analysis of membrane potential has been useful in studying neutrophil activation and can demonstrate subpopulations of cells with different polarization following stimulation.[37,38] Alterations in membrane potential can also be correlated with other tests of neutrophil function, such as chemotaxis, and may have potential clinical usefulness.[39,40] Membrane potential measurements may also have utility in monitoring drug effects on normal and tumor cells.[41–43]

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